Virology **254,** 182–195 (1999)
Article ID viro.1998.9536, available online at http://www.idealibrary.com on IDEAL®

Mechanism of Interferon Action: Functional Characterization of Positive and Negative Regulatory Domains That Modulate Transcriptional Activation of the Human RNA-Dependent Protein Kinase *Pkr* Promoter

Kelli L. Kuhen[1] and Charles E. Samuel[2]

Department of Molecular, Cellular and Developmental Biology and Interdepartmental Biochemistry and Molecular Biology Graduate Program, University of California, Santa Barbara, California 93106

Received October 2, 1998; returned to author for revision October 26, 1998; accepted November 8, 1998

The PKR protein kinase is an important regulator of viral mRNA translation. A ~50-kb gene (*Pkr*) encodes the human PKR protein that is inducible by interferon (IFN). The *Pkr* promoter region has a novel 15-bp DNA element designated as KCS required for transcriptional activity that is located 4 bp upstream of a 13-bp IFN-stimulated response element (ISRE) that confers inducibility by type I IFN. We have carried out a systematic analysis of the 5′ flanking region of the human *Pkr* gene to define how the novel KCS element acts to affect basal as well as IFN-inducible transcription. Electrophoretic mobility shift analyses (EMSA) revealed that nuclear proteins bound selectively to the KCS element in a manner that was not dependent upon either IFN treatment or protein binding at the adjacent ISRE element. KCS protein binding activity *in vitro* correlated with activation of transcription *in vivo* in transient transfection assays. Competition and supershift EMSA assays revealed that multiple proteins were involved in bandshift complex formation with KCS, one of which was identified as factor Sp1. In addition to the positive regulatory domain containing the KCS and ISRE elements, a negative regulatory domain (NRD) was identified within a 40-bp region positioned ~400-bp upstream of the KCS and ISRE elements. Deletion and substitution mutations indicated that the NRD negatively affected *Pkr* transcription by a mechanism dependent upon the KCS element. These results define novel positive and negative regulatory domains within the *Pkr* promoter that function through the KCS element to affect basal and IFN-inducible transcription of *Pkr.*

INTRODUCTION

The RNA-dependent protein kinase (PKR)[3] is an interferon-inducible, cyclic AMP-independent protein serine/threonine kinase (Samuel, 1979, 1993; Sen and Lengyel, 1992; Clemens and Elia, 1997). PKR plays a central role in the regulation of protein synthesis in virus-infected and IFN-treated cells (Samuel, 1991; Clemens and Elia, 1997). Phosphorylation of the α subunit of eukaryotic protein synthesis initiation factor 2 on serine residue 51, a modification catalyzed by PKR (Samuel, 1993), leads to the inhibition of mRNA translation (Hershey, 1989). PKR is also involved in the modulation of cytokine signaling and transcriptional activation via the NF-κB and STAT factors (Kumar *et al.,* 1997; Wong *et al.,* 1997). Because of these varied and fundamental biochemical activities, PKR affects a range of biological processes. For example, PKR is implicated in the control of cell growth, differentiation, and death (Lengyel, 1993; Clemens and Elia, 1997) as well as the antiviral actions of IFN (Samuel, 1991). Expression of the *Pkr* gene and activation of the PKR enzyme are regulated in many different ways in animal cells. These regulatory strategies include the transcriptional induction of *Pkr* gene expression by IFN treatment (Meurs *et al.,* 1990; Thomis *et al.,* 1992; Tanaka and Samuel, 1994; Kuhen and Samuel, 1997), the translational inhibition of PKR protein synthesis by an autoregulatory mechanism (Thomis and Samuel, 1992; Barber *et al.,* 1993), posttranslational activation of PKR kinase activity by an RNA-dependent autophosphorylation (Samuel, 1979; Thomis and Samuel, 1993; Romano *et al.,* 1998), and posttranslational modulation of PKR activity via homomeric and heteromeric protein–protein interactions (Lee *et al.,* 1994; Cosentino *et al.,* 1995; Ortega *et al.,* 1996; Patel *et al.,* 1996; Benkirane *et al.,* 1997).

The occurrence of increased transcription of the *Pkr* gene in IFN-treated cells above the basal level of expression is firmly established from Northern gel blot and nuclear run-on analyses with human PKR cDNA

The sequences reported in this study have been deposited in the GenBank database. The accession number assigned to 5′-flanking genomic sequence of the human Pkr gene, including the promoter region, is U51035.

[1] Present address: School of Medicine, University of California, San Diego, La Jolla, CA 92093-0665.

[2] To whom reprint requests should be addressed. Fax: (805) 893-4724.

[3] Abbreviations: dsRNA, double-stranded RNA; eIF-2α, alpha subunit of protein synthesis initiation factor 2; EMSA, electrophoretic mobility shift assay; IFN, interferon; PKR, IFN-induced RNA-dependent protein kinase; *Pkr*, gene encoding the PKR kinase.

0042-6822/99 $30.00

probes (Meurs *et al.,* 1990; Thomis *et al.,* 1992) as well as transient transfection analyses with isolated promoters from the human and mouse Pkr genes (Tanaka and Samuel, 1994; Kuhen and Samuel, 1997; Kuhen *et al.,* 1998). The human gene encoding the PKR kinase spans ~50-kb on chromosome 2p with the mature transcript consisting of 17 exons, which encode a 551-amino acid protein (Kuhen *et al.,* 1996). Transient transfection analyses using reporter plasmid constructions possessing various 5′ flanking fragments of the human *Pkr* gene led to the identification of a functional TATA-less promoter that directs IFN-inducible transcription (Kuhen and Samuel, 1997). Two DNA elements required for optimal promoter activity were identified. One was a consensus and functional copy of the IFN-stimulated response element (ISRE) responsible for the inducibility of many different genes by type I IFNs (Darnell, 1997; Nguyen *et al.,* 1997). The other was a novel 15-bp element, which was required for both basal and IFN-inducible promoter activity (Kuhen and Samuel, 1997; Kuhen *et al.,* 1998). This second element, designated as KCS for kinase conserved sequence, is exactly conserved in sequence (5′ GGGAAGGCGGAGTCC 3′) between the human and mouse *Pkr* promoters and in position relative to the ISRE element (Tanaka and Samuel, 1994; Kuhen and Samuel, 1997). The KCS element is found 4 bp immediately upstream of the ISRE element in the *Pkr* promoters.

It is well established that STATs (Darnell, 1997) and IRFs (Nguyen *et al.,* 1997) activate transcription through the ISRE element. However, no information is yet available regarding how the novel KCS element functions to activate transcription of the *Pkr* gene. Now it is important to determine whether proteins bind selectively to the KCS element and, if so, whether the KCS protein binding activity is affected by IFN treatment or by proteins bound at the adjacent ISRE element. Additionally, it is important to establish whether the activity of the KCS element is modulated by regulatory elements present within flanking sequences in a manner independent of the ISRE element.

Because of the central role of PKR in the antiviral actions of interferons (Samuel, 1991; Vilcek and Sen, 1996) and the implicated roles of PKR in the control of cell growth and differentiation as well as apoptosis (Lengyel, 1993; Clemens and Elia, 1997), we have attempted to define the *cis*-acting sequences and *trans*-acting factors responsible for transcriptional control of the *Pkr* gene through the novel KCS element. In this communication, we describe the functional characterization of positive and negative regulatory domains within 5′ flanking sequences of the human *Pkr* gene that modulate basal and IFN-inducible promoter activity.

RESULTS

Deletion of the KCS element reduces *Pkr* promoter activity

To examine whether elements reside in upstream flanking sequences of the *Pkr* promoter that modulate KCS function, a series of 5′ deletion mutants were generated that either lacked or retained the KCS element (Fig. 1). The 3′ terminus of all the promoter constructs terminated at the *Sac*II site, 25 bp downstream of the ISRE element. The variable 5′ ends extended through 1.6 kb of upstream sequence (Fig. 1A). CAT reporter results, calculated as the average from at least three independent transient transfection experiments, are shown in Fig. 1B.

Comparison of pairs of *Pkr* promoter constructs, differing only in the presence or absence of the KCS element but otherwise equivalent, revealed that the KCS element enhanced both basal and IFN-inducible transcription (Fig. 1B). The enhanced transcription observed with constructs that included the KCS element was independent of the nature of the 5′ flanking sequences upstream of the KCS. Deletion of the KCS motif reduced the *Pkr* promoter activity of all members of the family of constructs that possessed increasing amounts of genomic 5′ flanking sequence upstream of KCS. These constructs varied from having no additional 5′ sequence in the case of the 63-bp P/Sa construct to ~1.5 kb of added 5′ sequence in the case of the 1.6-kb H/Sa construct (Fig. 1). Basal CAT activity was reduced from 3- to 10-fold for the family of KCS *del* constructs and approached background levels of activity. Activities in IFN-treated cells were likewise reduced in a comparable manner by deletion of the KCS element, from three- to sevenfold relative to the wild-type value, depending upon the 5′ flanking sequence present. As a negative control, the promoterless pCAT-Basic plasmid vector without inserted genomic DNA exhibited low CAT activity (<2% conversion). By contrast, the positive control plasmid pCAT-Control, which contains the simian virus 40 promoter and enhancer, displayed high CAT activity levels (>90% conversion) in the transient transfection assay. Neither pCAT plasmid vector, the promoter-less pCAT-Basic, or the SV40 pCAT-Control, showed IFN-inducibility of the CAT reporter (data not shown). These results suggest that optimal transcriptional activation of the human *Pkr* promoter requires the KCS element, both in untreated and IFN-α-treated human U cells. The combined presence of the ISRE and upstream sequences containing potential binding sites for positively acting factors including AP-1, AP-2, and Sp1 (Kuhen and Samuel, 1997) was unable to compensate for the loss of KCS function.

Regulation of KCS element function by upstream sequences

We earlier observed that the KCS substitution mutant TM, possessing the three nucleotide substitutions (C8A,

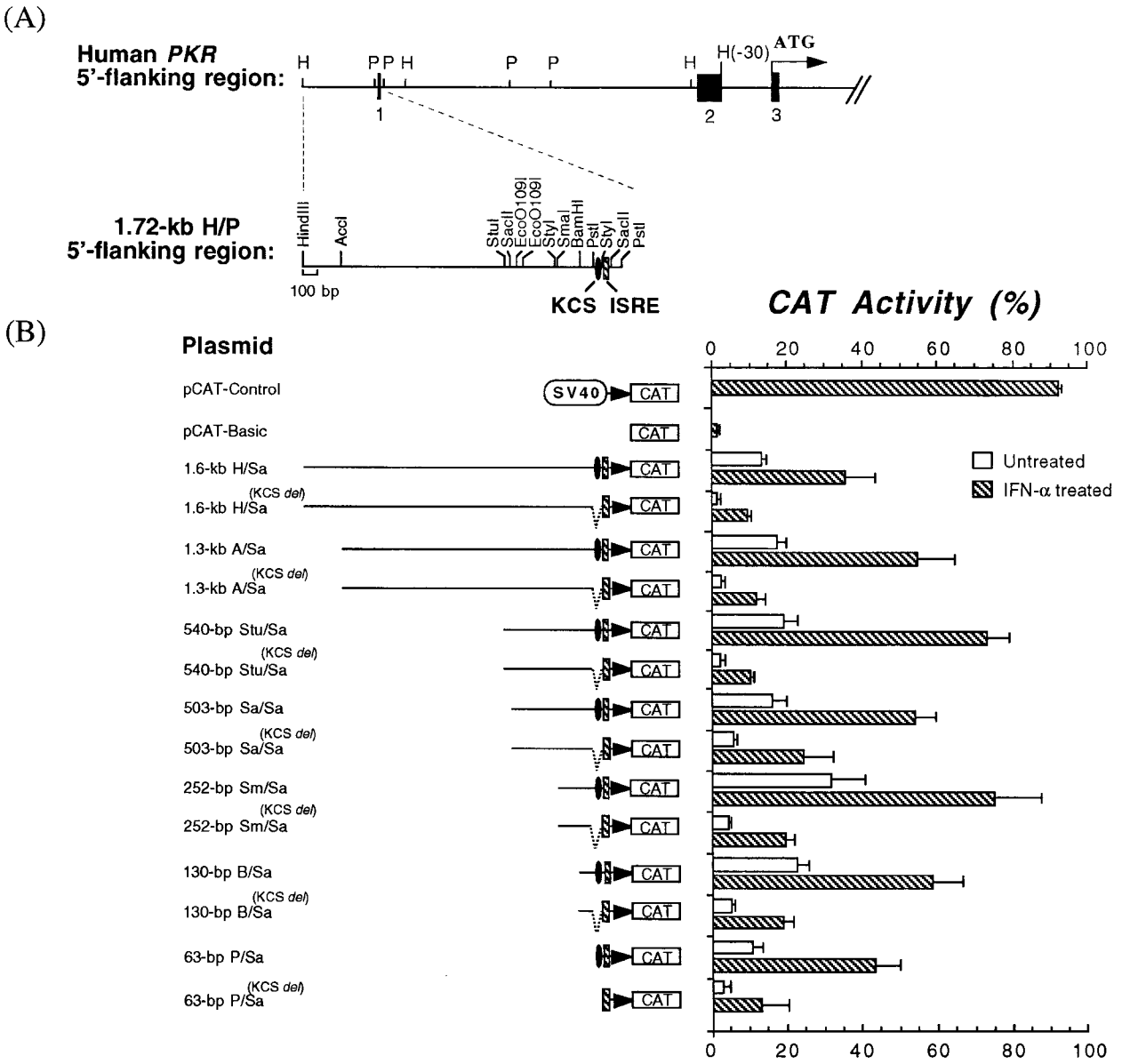

FIG. 1. Deletion of the KCS motif reduces *Pkr* promoter activity independent of the upstream 5′-flanking sequences. (A) The upper restriction map corresponds to the 5′ region of the human *Pkr* gene and includes the complete 5′ untranslated region present in exons 1, 2, and part of 3. The translation initiation site (ATG) for PKR is present in exon 3. The most 5′ part of the upper map, including the promoter region and exon 1, is shown in an expanded format by the detailed *lower* restriction map that corresponds to the 1.72-kb *Hin*dIII/*Pst*I promoter fragment. (B) The left side is a schematic representation of the CAT reporter plasmids constructed by the insertion of the indicated human *Pkr* promoter region restriction fragments into the promoterless pCAT-Basic plasmid. The KCS motif is depicted by the solid oval; deletion of the KCS motif is depicted by the dashed lines. The hatched rectangle corresponds the ISRE element. The right side shows the promoter activities observed in human amnion U cells transfected with the indicated wild-type and KCS *del* mutant reporter plasmids. Promoter activities of the various constructs are shown as percentage of the conversion of [^{14}C]chloramphenicol to the acetylated derivatives. To control for transfection efficiency, cells were cotransfected with the pRSV2-βgal construct as an internal reference. Open bars refer to cells left untreated, and hatched bars refer to cells treated with interferon. pCAT-Control, the CAT reporter gene linked to the simian virus 40 promoter and enhancer; pCAT-Basic, the promoterless plasmid vector without inserted human genomic DNA containing the *Pkr* promoter.

G9C, and G10T) within the KCS element, displayed greatly reduced promoter activity when examined in the background of the 503-bp Sa/Sa construct relative to the analogous wild-type 503-bp Sa/Sa construct (Kuhen and Samuel, 1997). We now have engineered the KCS substitution mutant TM into the same family of 5′ deletion constructs (Fig. 2) as those examined with the KCS deletion (Fig. 1). Surprisingly, the mutant phenotype of the KCS triple substitution mutant TM was dependent upon the length of the 5′ flanking genomic sequence upstream of the KCS element (Fig. 2). Those promoter constructs that retained additional 5′ genomic sequence upstream

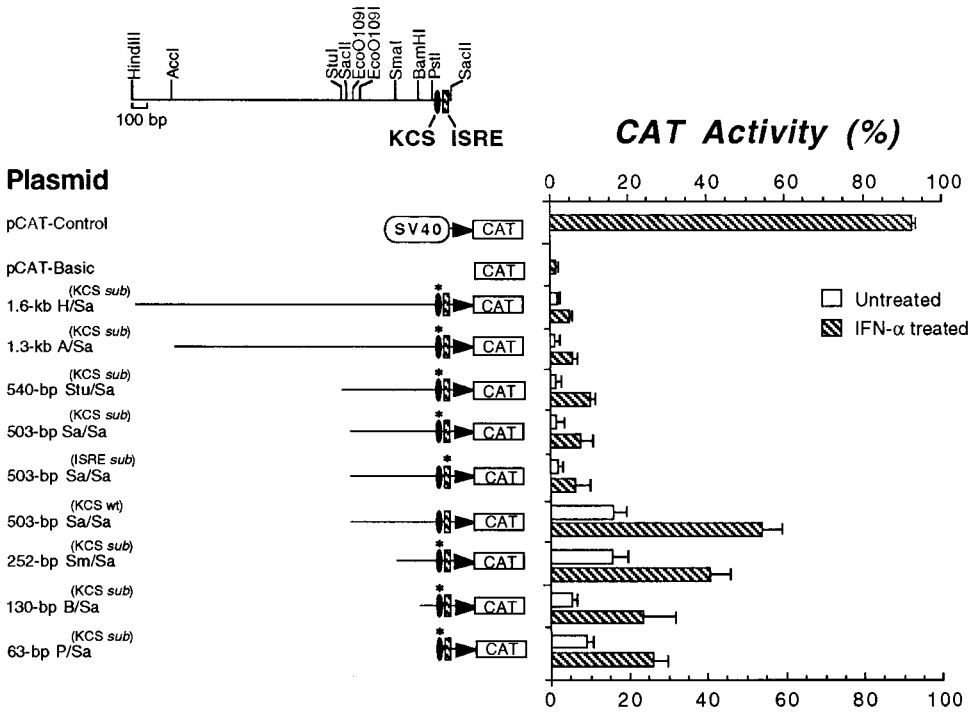

FIG. 2. Upstream 5′ flanking sequences regulate transcriptional activity mediated by the KCS element but not by the ISRE element. The left side is a schematic representation of the CAT reporter plasmids constructed by the insertion of the indicated human *Pkr* promoter region restriction fragments into the promoterless pCAT-Basic plasmid. The KCS element substitution mutant TM (C8A, G9C, G10T) is depicted by the asterisk above the solid oval; the hatched rectangle denotes the WT ISRE element. The ISRE element substitution mutant G8T is depicted by the asterisk above the hatched rectangle. The solid oval depicts the WT KCS motif. The right side shows the promoter activities observed in human amnion U cells transfected with the indicated KCS *sub* mutant reporter plasmids. Promoter activities of the constructs are shown as percentage of the conversion of [^{14}C]chloramphenicol to the acetylated derivatives. To control for transfection efficiency, cells were cotransfected with the pRSV2-βgal construct as an internal reference. Open bars refer to cells left untreated, and hatched bars refer to cells treated with IFN. pCAT-Control and pCAT-Basic are as described under the legend for Fig. 1.

of the *Sma*I site (503-bp Sa/Sa, 540-bp Stu/Sa, 1.3-kb A/Sa, and 1.6-kb H/Sa) showed a significant and consistent repression of transcription, both in the absence and presence of IFN treatment. Transcriptional repression ranged from a seven- to ninefold reduction measured by CAT reporter activity for the KCS *sub* mutant TM constructs in cells treated with IFN-α (Fig.2), relative to analogous wild-type KCS promoter constructs (Fig. 1). Relative basal CAT activity levels were reduced ~15-fold and approached the background value of the pCAT-basic construct (Fig. 2). A partial derepression of the negative effect that the KCS triple substitution mutation had on *Pkr* promoter activity was observed when the 5′ flanking upstream sequence terminated at or downstream of the *Sma*I site, as illustrated by the 252-bp Sm/Sa, 130-bp B/Sa, and 63-bp P/Sa constructs. Both basal and cytokine-induced levels of CAT activity were similarly affected. These results suggest that KCS function is regulated, at least in part, by elements present in the upstream flanking sequences.

To ascertain if the negative regulatory effect exerted by 5′ flanking sequences upstream of the KCS was specific for the KCS element or whether the upstream sequences also affected transcriptional events mediated by the ISRE element, the ISRE substitution G8T mutant was examined in the same family of constructs that possessed variable amounts of 5′ flanking sequence upstream of the KCS motif. The results revealed that the 5′ flanking sequences upstream of the KCS did not significantly affect ISRE element function (data not shown). The ISRE *sub* mutant displayed low activity with a wild-type KCS, independent of the length of 5′ flanking sequence upstream of the KCS motif. The IFN-inducible promoter activities of the KCS *sub* TM mutant and an ISRE *sub* G8T mutant were comparable and low in the 503-bp Sa/Sa promoter background, relative to the wild-type 503-bp Sa/Sa construct (Fig. 2).

Localization of an upstream KCS-specific regulatory region

Because the analysis of the nested 5′ deletion mutants indicated that sequences upstream of the *Sma*I site affected the function of the KCS element (Fig. 2), internal deletion mutants were generated in this region (Fig. 3). When the internal 210-bp *Eco*O109I(5′) through *Sma*I region from the 503-bp Sa/Sa parent promoter construct was removed, a partial derepression of the negative

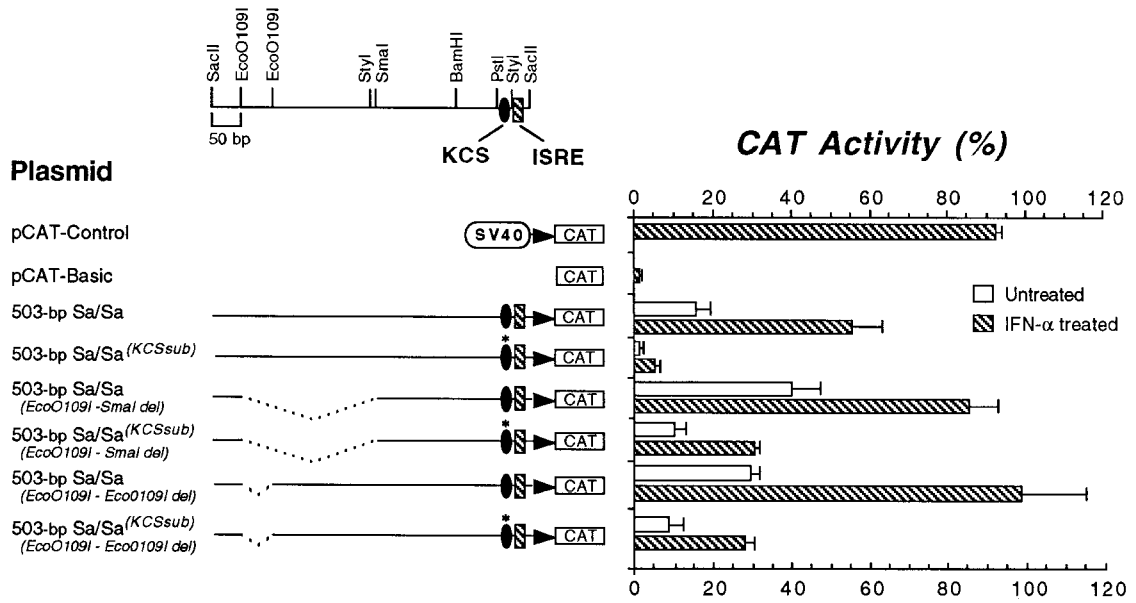

FIG. 3. Localization of a KCS-specific negative regulatory domain. The schematic representation (left side) summarizes the deletion mutant promoter constructs derived from the 503 bp Sa/Sa *Pkr* promoter fragment inserted into the promoterless pCAT-Basic plasmid vector. The WT KCS motif is depicted by the solid oval; the KCS substitution mutant TM (C8A, G9C, G10T) is depicted by the asterisk above the solid oval. The hatched rectangle denotes the WT ISRE element. The right side shows the promoter activities observed in human amnion U cells transfected with the indicated 503 bp Sa/Sa *PKR* promoter CAT reporter plasmids. Activities of deletion constructs, both WT and KCS *sub,* are shown as percentage of the conversion of [^{14}C]chloramphenicol to the acetylated derivatives. To control for transfection efficiency, cells were cotransfected with the pRSV2-βgal construct as an internal reference. Open bars refer to cells left untreated, and hatched bars refer to cells treated with interferon. pCAT-Control and pCAT-Basic are as described under the legend for Fig. 1.

effect observed with the KCS substitution mutant was observed. Deletion of only the 40-bp *Eco*O109I-*Eco*O109I fragment from the 503-bp Sa/Sa parent construct gave results similar to those observed with the larger *Eco*O109I-*Sma*I deletion. Both the constitutive and the IFN-α-inducible CAT activity levels were higher in cells transfected with KCS mutant constructs that lacked the *Eco*O109I-*Eco*O109I fragment region. Interestingly, wild-type promoter activity was also increased by removal of the *Eco*O109I fragment region from the reporter constructs. These results suggest this region of the *Pkr* promoter possesses negative regulatory element(s) that act to silence *Pkr* promoter function by modulating transcriptional events dependent upon the KCS motif.

Proteins selectively bind the KCS element

Results of transient transfection analyses of the *Pkr* promoter constructs suggest that the KCS element functions as a positive regulatory domain (Figs. 1–3). The data are consistent with the role of KCS as a constitutive activator element and that KCS is not involved in mediating IFN inducibility. To examine whether protein factors are present in human cells that bind selectively to the KCS element, EMSA analyses were conducted. A wild-type KCS synthetic oligomer was used as the probe. When incubated with nuclear extracts prepared from untreated human U cells, the ^{32}P-labeled KCS probe gave two major complexes designated KBP for KCS binding protein complex, and NS for nonspecific complex (Fig. 4). A third bandshift complex of minor intensity, with a relative intermediate mobility between KBP and NS, was also detected (KBP′). Formation of the KBP complexes was not dependent on IFN treatment (data not shown; see also Fig. 7).

Specificity of protein binding to the KCS probe was demonstrated by competition analysis. A 100-fold molar excess of unlabeled KCS oligo efficiently competed for factor binding to the ^{32}P-labeled KCS oligo probe (Fig. 4, lane 3). By contrast, a 250-fold molar excess of the unlabeled ISRE oligo did not significantly reduce the amount of the KBP complex formed with the KCS oligo probe (Fig. 4, lane 8). The formation of the faster migrating NS bandshift complex was competed comparably by a 100-fold molar excess of either the KCS or the ISRE oligo, suggesting a nonspecific (NS) DNA-binding activity. These results demonstrate that nuclear proteins can selectively bind the KCS element *in vitro*.

KCS protein binding *in vitro* correlates with transcriptional activation *in vivo*

To examine whether the formation of the KBP bandshift complex *in vitro* correlates with KCS-mediated transcriptional activity *in vivo* in the transient transfection assay, two KCS single bp substitution mutants were generated, and their activities compared to that of the wild-type KCS in transient transfections and EMSA as-

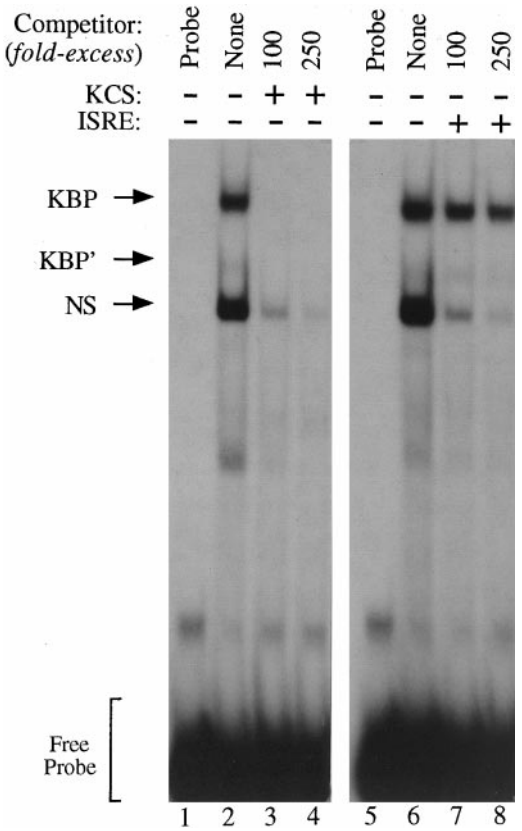

FIG. 4. Detection of KCS-binding proteins by electrophoretic mobility shift assay (EMSA). KCS complexes were detected in nuclear extracts prepared from human amnion U cells. EMSA was carried out using a synthetic dsDNA oligomer probe, possessing the KCS element, that corresponded to nt 614–637 of the human *Pkr* promoter. Nuclear extracts (9 μg of protein) were incubated with ^{32}P-labeled KCS oligomer probe in the absence of competitors (lanes 2 and 6) or presence of either a 100- or 250-fold molar excess of the unlabeled wild-type 24-mer KCS oligomer (lanes 3 and 4) or wild-type 23-mer ISRE oligomer (lanes 7 and 8). The KCS-binding protein complexes (KBP, KBP′) are indicated as well as the unbound free probe. NS, nonspecific binding.

says. For analysis of transcriptional activity, the two single bp KCS substitution mutants, KCS(mt6A) and KCS(mt9T), were introduced into the background of the 503-bp Sa/Sa *PKR* promoter CAT reporter construct. The promoter activities of the resultant KCS(mt6A) and KCS(mt9T) constructs were substantially reduced relative to the wild-type parent 503-bp Sa/Sa construct (Fig. 5). The activities, both basal and IFN-inducible, of the KCS(mt6A) and KCS(mt9T) mutants were low, and comparable to that of the 503-bp Sa/Sa TM mutant construct (Fig. 5). These data further support the notion that the KCS element has a positive regulatory role in the transcription of the *Pkr* gene.

The ability of double-stranded KCS(mt6A) and KCS(mt9T) mutant oligomers to compete the formation of the KBP complex formation was examined at varying oligomer concentrations, ranging from a 5- to 100-fold molar excess of the unlabeled competitor over the ^{32}P-labeled KCS(WT) probe. As shown in Fig. 6, the

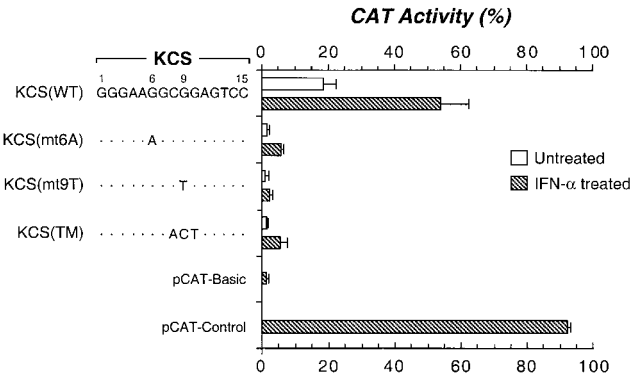

FIG. 5. Single nucleotide substitutions within the KCS element reduce *Pkr* promoter activity. Mutant promoter constructs were derived from the 503-bp Sa/Sa wild-type parent plasmid. KCS(WT), the 15-bp wild-type KCS element in the 503-bp Sa/Sa construct. For KCS(mt6A) and KCS(mt9T), the indicated G6 to A6, or G9 to T9, substitution was introduced within the KCS motif. KCS(TM), the triple substitution KCS mutant (C8A, G9C, G10T). Promoter activities of the constructs are shown as percentage of the conversion of [^{14}C]chloramphenicol to the acetylated derivatives. To control for transfection efficiency, cells were cotransfected with the pRSV2-βgal construct as an internal reference. Open bars refer to cells left untreated, and hatched bars refer to cells treated with interferon. pCAT-Control and pCAT-Basic are as described under the legend for Fig. 1.

KCS(mt6A) oligo had no apparent competitive effect on KBP complex formation, whereas KCS(mt9T) interestingly was a weak competitor when examined at high molar excess. The data point which corresponds to a 100-fold molar excess of unlabeled competitor represents an average of six experiments (Fig. 6). Typically, ~55% of the KCS-specific KBP bandshift complex remained even

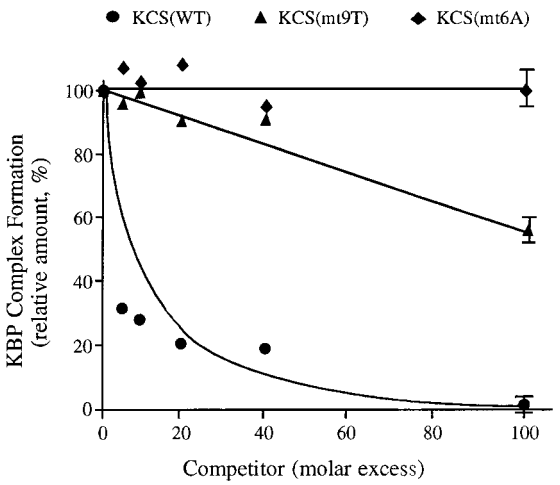

FIG. 6. Effect of competitor oligonucleotide concentration on KBP complex formation measured by EMSA. Nuclear extract prepared from human amnion U cells was incubated with ^{32}P-labeled KCS(WT) oligonucleotide probe, either in the absence of competitors or presence of the indicated concentration of unlabeled competitor as follows: KCS(WT), KCS(mt6A) mutant, or KCS(mt9T) mutant. A Bio-Rad GS525 molecular imager system was used for quantification of the amount of KBP complex formed.

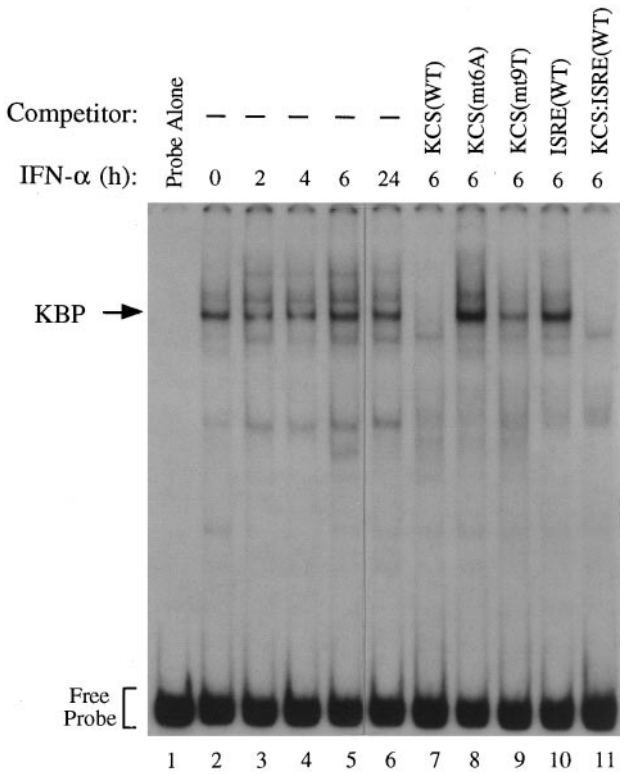

FIG. 7. Effect of interferon treatment on KCS-binding protein complex formation detected by EMSA. EMSA was carried out with the 130-bp B/Sa DNA fragment that possesses both the KCS and ISRE elements. Nuclear extracts prepared from human amnion U cells (9 μg of protein), either untreated or treated with IFN-α, were incubated with the ^{32}P-labeled 130-bp B/Sa DNA fragment. IFN treatment ranged from 0 (lane 2) to 24 h (lanes 3–6), as indicated. Specificity of binding to the fragment was determined by competition (lanes 7–11), using extracts prepared from cells treated for 6 h with IFN-α. ^{32}P-labeled *Bam*HI/*Sac*I probe was examined in the absence of competitors (lanes 1–6) or presence of a 100-fold molar excess of the indicated dsDNA synthetic oligomers as indicated: KCS(WT) (lane 7), KCS(mt6A) mutant (lane 8), KCS(mt9T) mutant (lane 9), ISRE(WT) (lane 10), or KCS:ISRE(WT) (lane 11). KBP, KCS-binding protein complex.

when a 100-fold molar excess of the KCS(mt9T) oligomer was added. By comparison, the KCS(mt6A) oligomer did not compete the binding of the ^{32}P-labeled KCS(WT) probe. The wild-type KCS oligomer was an efficient competitor; a 100-fold molar excess of KCS(WT) completely prevented bandshift formation (Fig. 6). These results indicate that KBP complex formation detected by EMSA correlates with transcriptional activation measured by transient transfection of a CAT reporter, and that more than one protein component may be responsible for mediating KCS function.

The 130-bp *Bam*HI/*Sac*I fragment from the 5′ flanking region, which possesses both the KCS and ISRE regulatory elements, supported CAT gene transcription in an IFN-inducible manner (Fig. 1). When this 130-bp natural promoter fragment was examined as the probe in the EMSA assay, a bandshift complex was detected (Fig. 7) that possessed characteristics comparable to those observed for the chemically synthesized KCS(WT) probe (Fig. 6). The major KBP bandshift complex observed with the ^{32}P-labeled *Bam*HI/*Sac*I probe was efficiently competed, both with the KCS(WT) and the KCS:ISRE oligomers at 100-fold molar excess (Fig. 7, lanes 7 and 11, respectively). However, the ISRE(WT) and the mutant KCS(mt6A) oligos did not compete (Fig.7, lanes 8 and 10) under similar conditions, and the KCS(mt9T) mutant oligomer was only a weak competitor (Fig. 7, lane 9). Formation of the KBP complex was comparable with nuclear extracts isolated from U cells that had been left untreated as compared to cells treated with a saturating concentration of IFN-α during a 24-h period (Fig. 7, lanes 2–6).

The 5′ and 3′ halves of the KCS element each mediate transcription factor binding

The observation that the KCS(mt9T) oligomer partially competed for protein binding to the wild-type KCS probe in EMSA analysis (Figs. 6 and 7) suggested that the KCS(mt9T) oligo retained limited protein binding activity even though the substitution of a T for G at nt position 9 of the KCS element inhibited transcription measured with the CAT reporter (Fig. 5). Indeed, when the KCS(mt9T) mutant oligo was used as the probe in the EMSA assay, the ^{32}P-labeled KCS(mt9T) oligomer possessed the ability to bind in a specific manner factors constitutively present in nuclear extracts from U cells (Fig. 8A). Formation of the ^{32}P-labeled KCS(mt9T)-specific bandshift complex, designated KBP1, was competed completely by a 100-fold molar excess of either wild-type KCS(WT) or mutant KCS(mt9T) unlabeled oligomer (Fig. 8A, lanes 3 and 4). However, the mutant KCS(mt6A) and wild-type ISRE oligomers only partially competed complex formation with the KCS(mt9T) probe (Fig. 8A, lanes 5 and 6).

The finding that the ^{32}P-labeled KCS(mt9T) probe generated a minor bandshift relative to the wild-type KCS probe (Fig. 8A) suggested that a second KBP may possibly be involved in increasing the affinity of KBP1 for the KCS motif or alternatively that optimal binding by KBP1 may require the wild-type sequence at both positions 6 and 9 of the KCS motif. Therefore, the KCS(mt6A) oligomer was examined as the probe in an EMSA assay. As shown in Fig. 8A, the KCS(mt6A) probe formed a complex, designated herein as KCS-binding protein 2 or KBP2, that was competed by unlabeled KCS(WT), mutant KCS(mt6A), mutant KCS(mt9T), and ISRE(WT) oligomers (Fig. 8A, lanes 8–12). This result suggested either that KBP2 had a low affinity for KCS or that the KBP2 complex was nonspecific.

The Sp1 transcription factor is likely involved in mediating KCS function

The 5′ portion of the KCS element corresponding to the GGGAAGG sequence conforms to a low-affinity binding site for the Sp1 transcription factor (Kutoh *et al.*,

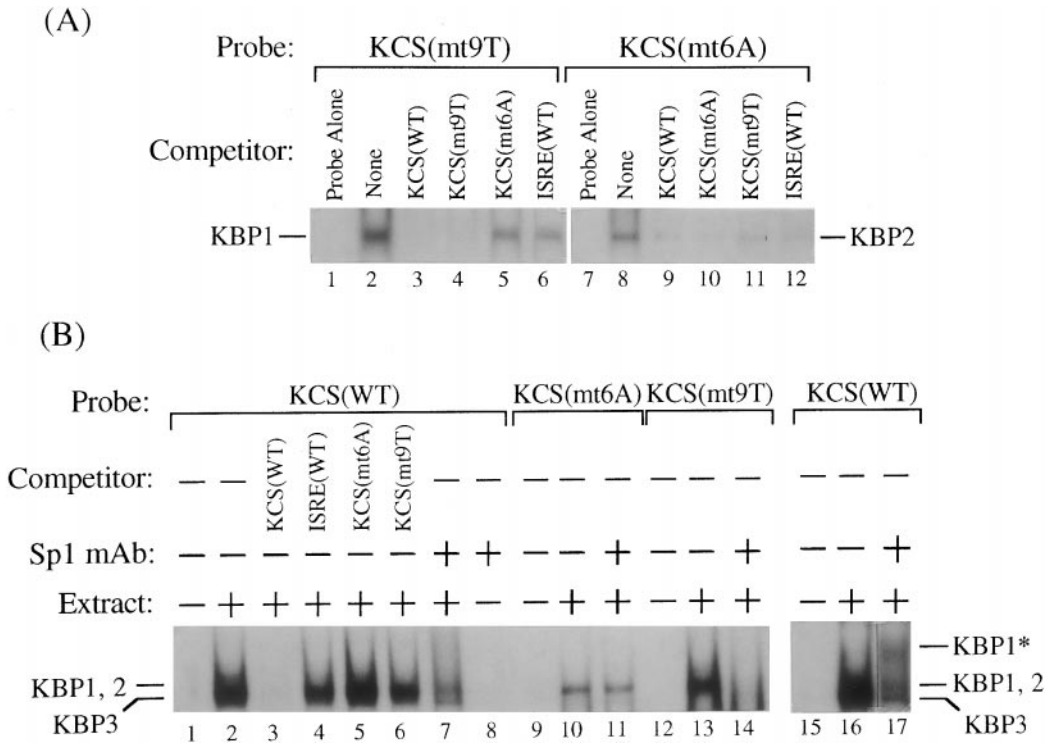

FIG. 8. Multiple proteins bind selectively to the KCS element as detected by EMSA. Nuclear extracts prepared from human amnion U cells (9 μg of protein) were incubated with either wild-type (WT) or mutant ^{32}P-labeled KCS probe. Competitors were present at 100-fold molar excess as indicated. (A) Probes were KCS(mt9T) (lanes 1–6) or KCS(mt6A) (lanes 7–12). KBP1, the single KCS binding protein specific for KCS(mt9T). KBP2, a second KCS DNA binding protein, specific for KCS(mt6A). (B) KCS wild-type probe (lanes 1–8), the KCS(mt6A) probe (lanes 9–11), or the KCS(mt9T) probe (lanes 12–14), examined in the presence (lanes 7, 8, 11, and 14) or absence of monoclonal antibody against Spl. The KBP1 (Spl), KBP2, and KBP3 complexes are indicated. Lanes 15–17, EMSA assay carried out as for lanes 1, 2, and 7, but the gel was overexposed to detect the supershifted complex (KBP1*).

1993). To determine whether the Sp1 factor binds to the KCS motif and potentially is a component of KBP1, U cell nuclear extracts were examined for bandshift activity with ^{32}P-labeled wild-type KCS probe in the presence of a human Spl monoclonal antibody added prior to addition of the probe. Addition of Sp1 antibody greatly reduced KBP complex formation with the wild-type KCS oligo (Fig. 8B, lane 7). Longer autoradiogram exposure times revealed that a supershift complex was generated (KBPl*) when the Sp1 antibody was present (Fig. 8B, lane 17). Curiously, two bandshift complexes remain, designated KBP2 and KBP3. When U cell nuclear extracts were pre-incubated with the Spl antibody and then either the KCS(mt6A) or the KCS(mt9T) ^{32}P-labeled-probe was added, the Spl antibody abrogated KBP1 complex formation with the KCS(mt9T) probe (Fig. 8B, lane 14) but not KBP2 complex formation with the KCS(mt6A) probe (Fig. 8B, lane 11). These results indicate that two or more different proteins are involved in selective complex formation with the KCS element to generate complexes that display a similar gel mobility under the assay conditions employed. The results also revealed the bandshift complex generated with the KCS(mt6A) probe does not involve Sp1 but rather likely represents a low-affinity KCS bandshift. By contrast, the KCS(mt9T)-specific shift likely includes an Sp1-like transcription factor, consistent with the retention of an intact Sp1 binding site within the mutant KCS(mt9T) oligomer.

Altered spacing between the KCS and ISRE elements does not affect *Pkr* promoter activity whereas duplication of the elements enhances basal activity

Because of the close proximity of the KCS and ISRE elements to each other and because the spacing of the 4 bp between the elements is exactly conserved between the human (Kuhen and Samuel, 1997) and the mouse (Tanaka and Samuel, 1994) *Pkr* promoters, it is conceivable that protein factors that bind to the two elements may interact with each other. To begin to address this possibility, the spacing between the two adjacent elements was increased by an increment of 4 bp. Because 10 bp comprise a full turn of a DNA helix and 5 bp constitute a half-turn (Werner *et al.,* 1996), it was anticipated that an insertion of 4 bp might cause DNA-binding proteins to no longer contact their cognate sites on the same face of the helix. Thus the insertion might alter the potential for protein–protein interactions. As shown in Fig. 9, the increased spacing by 4 bp between the KCS and ISRE elements did not significantly affect

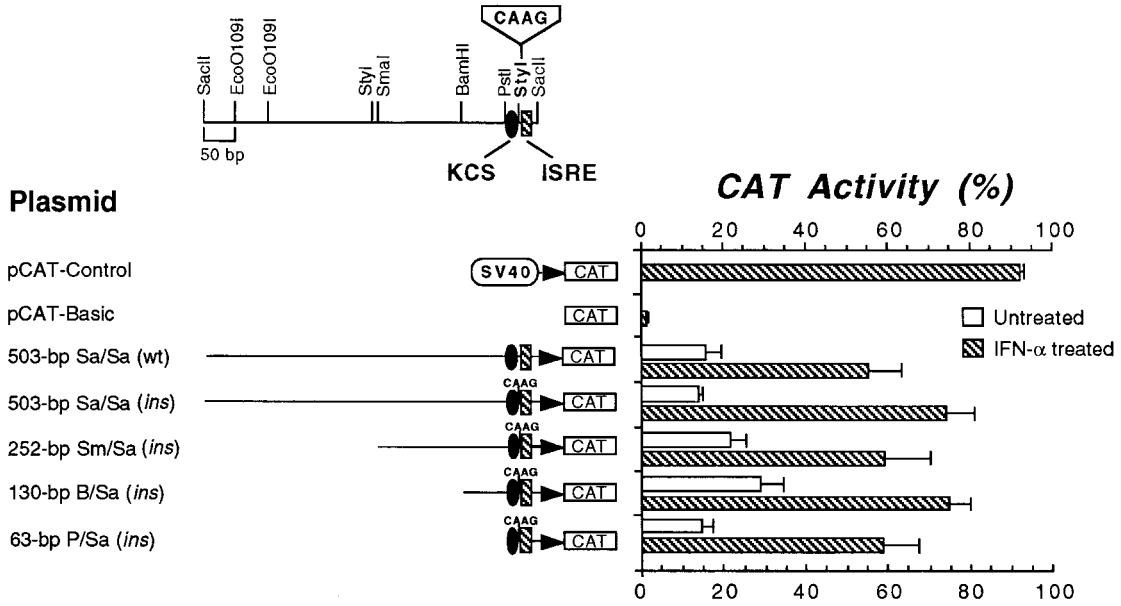

FIG. 9. Increased spacing between the KCS and ISRE motifs does not affect *Pkr* promoter activity. The schematic representation (left side) summarizes the CAT reporter constructs that possess an increased spacing between the KCS element and the ISRE element by insertion (*ins*) of the four nt "CAAG" at the *Sty*I site between the two elements. The solid oval denotes the WT KCS motif; the hatched rectangle denotes the WT ISRE element. The right side shows the promoter activities observed in human amnion U cells transfected with the indicated *Pkr* promoter CAT reporter plasmids. Activities are shown as percentage of the conversion of [^{14}C]chloramphenicol to the acetylated derivatives. To control for transfection efficiency, cells were cotransfected with the pRSV2-βgal construct as an internal reference. Open bars refer to cells left untreated, and hatched bars refer to cells treated with interferon. pCAT-Control and pCAT-Basic are as described under the legend for Fig. 1.

Pkr promoter activity. The family of 5′ nested deletion constructs possessing the insertion (*ins*) mutation displayed activities comparable to the 503 Sa/Sa (wt) reference construct (Fig. 9). These results suggest that KCS- and ISRE-binding proteins and their cognate DNA-binding sites likely function in separate and distinct manners to activate efficient transcription of the human *Pkr* gene.

The results shown in Fig. 1 revealed that the 63-bp fragment of the 5′ flanking region of the *Pkr* gene that possessed the KCS and ISRE elements displayed IFN-inducible promoter activity in the transient transfection CAT reporter assay. As shown in Fig. 10, this activity was dependent upon the presence of the KCS element because the 40-bp St/Sa construct that lacks the KCS element showed greatly reduced promoter activity relative to the 63-bp P/Sa construct. However, the chemically synthesized KCS:ISRE promoter designed to contain only the KCS and ISRE elements, flanked by the native 5′ and an engineered 3′ *Pst*I restriction site, showed IFN-inducible promoter activity. The IFN-inducible and the basal promoter activities of the synthetic KCS:ISRE promoter were comparable to those of the natural 63-bp P/Sa construct (Fig. 10). Surprisingly, the (KCS:ISRE)x2 construct that possessed tandem copies of the synthetic KCS:ISRE DNA fragment showed very strong basal promoter activity that was not significantly further increased by IFN treatment (Fig. 10). The increase in basal promoter activity observed upon duplication of the KCS element is consistent with the conclusion that KCS possesses a constitutive activator function.

DISCUSSION

This investigation was undertaken to gain insight into the mechanism by which the novel DNA element present within the *Pkr* promoter, designated as KCS (Kuhen and Samuel, 1997), affects basal as well as IFN-inducible transcription of the RNA-dependent protein kinase PKR. Although PKR is an IFN-inducible enzyme that is part of the antiviral response (Samuel, 1991), significant basal expression of PKR is often observed in human cells (Samuel, 1979; Thomis *et al.,* 1992; Clemens and Elia, 1997). Five important points emerged from our studies of the 5′ flanking region of the human *Pkr* gene that provide new insights regarding the manner in which the activity of the *Pkr* promoter is controlled as well as the role of the KCS element in the modulation of *Pkr* promoter activity. First, the KCS element functions as a positive regulatory element that is required both for basal and for IFN-inducible transcriptional activity of the human *Pkr* promoter. Second, nuclear proteins selectively bind to the KCS element in a manner that is not dependent upon IFN treatment. Third, KCS protein binding activity *in vitro* correlates with activation of transcription *in vivo* in a transient transfection assay. Fourth, the protein complexes that interact with the KCS element can be distinguished by competition EMSA assays from those that

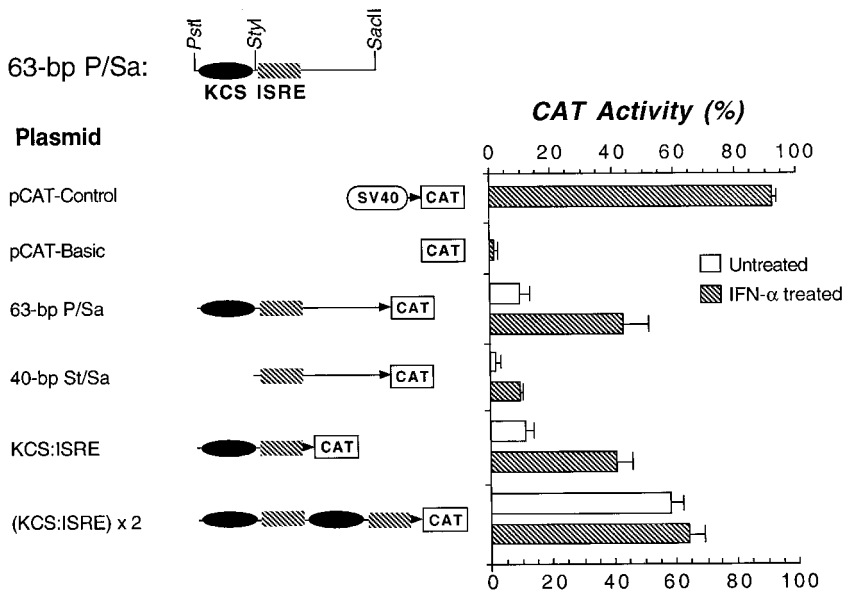

FIG. 10. Promoter activity of chemically synthesized KCS and ISRE elements in single and tandem copies. The schematic representation (left side) summarizes the CAT reporter constructs that possess either the naturally occurring 63-bp *Pst*I-*Sac*II fragment and the 40-bp *Sty*I-*Sac*II fragment from the 5′ flanking region of the *Pkr* gene, or a synthetic promoter fragement, inserted into the pCAT-Basic vector. The KCS:ISRE construct consists of a chemically synthesized DNA fragment possessing only the KCS and ISRE elements flanked by the native 5′ and engineered 3′ *Pst*I sites. The (KCS:ISRE)x2 construct possesses tandem copies of the synthetic KCS:ISRE fragment inserted into pCAT-Basic. pCAT-Control and pCAT-Basic are as described under the legend for Fig. 1.

interact at the adjacent ISRE element. Three complexes formed with KCS, involving proteins designated KBP1, KBP2, and KBP3. KBP1 appears equivalent to the transcription factor Sp1. Fifth, a negative regulatory domain (NRD) is present within a 40-bp region located ~400 bp upstream of the KCS and ISRE elements. The NRD domain negatively affects *Pkr* transcription by a mechanism dependent upon the KCS element. The schematic diagram shown in Fig. 11 provides a summary of the relative organization of the KCS, ISRE, and NRD DNA elements within the promoter region of the human *Pkr* gene, and the cognate *trans*-acting proteins bound by them.

The KCS element was specifically bound by proteins present in the nuclear extracts prepared from human cells, designated as KBPs for KCS binding proteins. The binding of KBP proteins to the KCS element measured by EMSA *in vitro* correlated with the activity of the promoter in transfected cells measured with the CAT reporter. Systematic mutational analysis of the 15-bp KCS element permitted the identification of the nucleotide positions within the element that are most essential for transcriptional activity. From the analysis of a total of 45 KCS single substitution mutants in the background of the parent 503 Sa/Sa promoter construct that contains the wild-type ISRE element, it was found that substitutions at either position 6 or 9 greatly reduce promoter activity (Kuhen *et al.,* 1998). Two 503 promoter mutants, KCS(mt6A), which possesses an A in place of the G at position 6, and KCS(mt9T), which possesses a T in place of the wild-type G at position 9, lacked appreciable promoter activity in human cells transiently transfected with CAT reporter constructs. The formation of specific KBP bandshift complexes were readily detectable by EMSA with the KCS(WT) element as the ^{32}P-labeled oligonucleotide probe. Although such complex formation was efficiently competed with the unlabeled KCS(WT) oligomer,

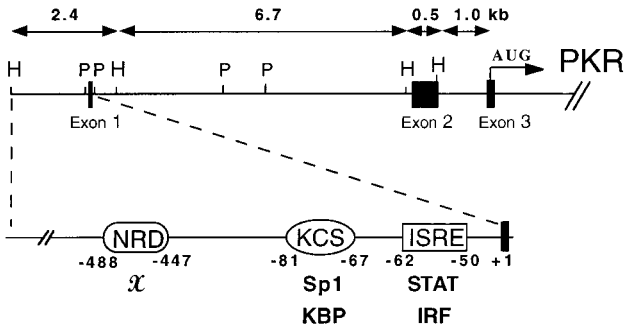

FIG. 11. Schematic summary of the organization of the promoter region of the human *Pkr* gene. The upper restriction map corresponds to the 5′ flanking region of the human *Pkr* gene. The solid squares correspond to exons 1, 2, and part of 3. The translation initiation site (ATG) for PKR is present in exon 3. The most 5′ part of the upper map corresponding to the promoter region is shown in an expanded format by the detailed lower schematic that depicts positive (KCS, ISRE) and negative (NRD) regulatory elements. ISRE, the 13-bp IFN-stimulated response element (ISRE) responsible for IFN inducibility to which STAT and IRF factors bind; KCS, the novel 15-bp element required for optimal promoter activity to which Sp1 and as yet unidentified additional proteins (KBP) bind; and NRD, the negative regulatory domain corresponding to the 40-bp *Eco*O109I-*Eco*O109I fragment upstream of the KCS and ISRE elements.

it was not effectively competed with either the KCS(mt6A), the KCS(mt9T) or the ISRE(WT) oligomer when examined as the unlabeled competitor. Both the basal and the IFN-inducible promoter activities of the single substitution KCS promoter mutants KCS(mt6A) and KCS(mt9T) in the 503 promoter background were effectively abolished, consistent with the impaired and reduced protein binding activity of the KCS(mt6A) and KCS(mt9T) mutant oligomers, respectively, revealed by their inability to compete in the EMSA assay (Fig. 6).

Our results obtained from the analysis of deletion and substitution mutants suggest that the constitutive activator function of the KCS element may be regulated by both activator and repressor proteins. The NRD regulatory region, localized to a 40-bp sequence ~400 bp upstream of KCS, was observed to exert a silencing effect on *Pkr* gene transcription through involvement of the 5′ half of the KCS element. That transcriptional silencing is observed only in the presence of both the NRD region (Fig. 3) and the KCS element and more specifically the 5′ half of the KCS (Fig. 2), it is conceivable that modulation of KCS function by the 40-bp NRD may involve a repressor protein that impairs the function of a KCS activator protein. This might occur indirectly by protein–protein interactions between the putative repressor protein bound at the NRD and a KCS activator protein or directly by protein–DNA interactions involving a repressor bound at the KCS activator site but stabilized through NRD protein interactions. Interestingly, we have observed that two KCS substitution mutants (G2A and A5G) possess slightly increased promoter activity, both basal and inducible (Kuhen *et al.,* 1998), which is consistent with negative regulation of KCS function through the 5′ half of KCS.

The Pkr promoter region is G/C rich and contains potential Sp1 binding sites immediately 5′ of the ISRE, including a consensus Sp1 site (GGGCGG) and also a low-affinity Sp1 site (GGGAAGG) that corresponds to the 5′ half of the KCS element. Interestingly, a similar arrangement of the ISRE element and Sp1 sites occurs in the IFN-inducible promoter of the 6–16 gene (Porter *et al.,* 1988). The EMSA results obtained using KCS mutant oligos KCS(mt6A) and KCS(mt9T), and a monoclonal antibody to Sp1, are consistent with the notion that Sp1 is equivalent to KBP1 and that the binding of the KCS element by the activator proteins KBP1 and KBP2 positively affects the constitutive level of *Pkr* gene expression. It is well established that Sp1 factors can stimulate promoter-specific activation of RNA polymerase II transcription by mechanisms involving cooperative interactions with other transcription factors (Kadonaga *et al.,* 1986; Kutoh *et al.,* 1993). Interestingly, it has been reported that Stat1 function depends on transcriptional synergy with Sp1 (Look *et al.,* 1995).

Two important families of transcription regulators involved in modulation of the transcriptional activity of IFN-inducible promoters through the ISRE element are the STATs (Darnell, 1997) and IRFs (Nguyen *et al.,* 1997). IFN treatment mediates phosphorylation of Stat1 and Stat2 in the cytoplasm and their subsequent heterodimer formation and nuclear translocation and transcriptional activation via binding to the *cis*-acting ISRE element (Darnell, 1997). In the case of IFN-α, the multiprotein transcriptional activator complex ISGF3 composed of Stat1, Stat2, and p48 binds to the ISRE element. The ISRE element also binds members of the IFN regulatory factor (IRF) family of proteins to which the p48 DNA-binding protein component of the ISGF3 complex belongs (Nguyen *et al.,* 1997). In addition to interaction of STATs and IRFs with the ISRE DNA element, the STAT and IRF proteins also interact with other DNA-binding proteins such as Sp1, CBP, PU1, and p300 (Look *et al.,* 1995; Darnell, 1997; Nguyen *et al.,* 1997). Our results reveal that two additional DNA elements affect the ISRE-dependent (Kuhen and Samuel, 1997) transcription of the human Pkr gene: the positively acting KCS element to which Sp1 and perhaps two or more other proteins bind and the upstream negatively acting NRD, which mediates its function in a manner dependent upon the KCS element. The KCS element affects both basal and IFN-inducible transcription of the *Pkr* gene. However, because the ISRE oligomer did not compete with the complex formation generated with the KCS probe detected by EMSA unless the KCS element sequence was also present with the ISRE sequence in the competitor, it appears that protein binding at the KCS element occurs independently of proteins such as STATs and IRFs bound at the ISRE element.

It is now of utmost importance to attempt to identify the KBP2 and KBP3 protein factors that interact with the KCS element and to determine how these proteins function with Sp1 (KBP1) to affect the transcriptional activity of the *Pkr* promoter through the KCS element. In addition, the elucidation of the biochemical mechanism by which the negative regulatory domain located upstream of the KCS element affects *Pkr* promoter activity, and whether the effects of NRD differ under varying conditions of cell growth and cytokine treatment, is of central importance.

MATERIALS AND METHODS

Construction of reporter gene plasmids

The pCAT-Basic promoterless plasmid (Promega) containing the chloramphenicol acetyltransferase (CAT) gene was used for construction of the *PKR* promoter:CAT reporter gene plasmids. The 503-bp Sa/Sa human *PKR* promoter construct in the pCAT-Basic promoterless plasmid, the single nucleotide substitution mutation within the ISRE element (G8T) of the 503-bp Sa/Sa construct, and the triple substitution mutation within the KCS motif (C8A, G9C, G10T) of the 503-bp Sa/Sa construct were as previously described (Kuhen and Samuel, 1997). The two

mutant KCS PCR products, each possessing a single bp substitution within the KCS element, were subcloned into the 503-bp Sa/Sa promoter parent background by exchanging the *Pst*I (*PKR*)-*Xba*I (pCAT-MCS) region for the *Pst*I-*Sac*II-digested PCR product possessing the KCS mutation. The 503-bp Sa/Sa promoter constructs possessing internal deletions were generated by removal of the internal restriction fragment with the appropriate endonucleases, and then each was blunted and ligated using T4 DNA polymerase and T4 DNA ligase, respectively. The promoter constructs that possessed the 4-bp CAAG insertion between the KCS and ISRE elements were generated by digestion of the indicated promoter fragment in the pBluescript vector with *Sty*I restriction endonuclease. pBluescript lacks vector *Sty*I sites, unlike the pCAT-Basic vector, which possesses two *Sty*I sites. The *Sty*I overhangs of the linearized pBluescript-*PKR* construct were then filled in using Klenow fragment polymerase to generate the insertion and then circularized using T4 DNA ligase. The insertion mutant promoter fragments were subcloned from pBluescript into the pCAT-Basic promoterless plasmid for expression analyses. All subcloning procedures were carried out following standard cloning procedures (Sambrook *et al.,* 1989), and resultant constructs were confirmed by restriction enzyme analysis and/or sequence analysis. Plasmid subclones were sequenced by the Sanger dideoxynucleotide procedure to verify the construction (Sanger *et al.,* 1977).

Oligonucleotide-directed mutagenesis

Single nucleotide substitutions within the KCS element of the human *Pkr* 5′ flanking region (Kuhen and Samuel, 1997) were generated using a PCR-based method for site-directed mutagenesis. The PCR products were engineered to possess appropriate restriction sites that would facilitate subcloning of the mutated KCS element into the 503-bp Sa/Sa human *PKR* promoter parent construct. The PCR (+) primers were the oligonucleotides used for mutagenesis; primers possessed the 15-nt KCS motif and the naturally occurring and partially overlapping 5′ flanking *Pst*I restriction enzyme site, which is indicated in italic type: 5′*CTGCAG*GGAAGGCGGAGTCC3′. The KCS(mt6A) mutant at position 6 was generated with an oligomer of the sequence 5′*CTGCAG*GGAA**A**GCGGAGTCC3′ and the KCS(mt9T) with the oligomer 5′*CTGCAG*GGAAGGC**T**GAGTCC3′, where the underlined bold type indicates the site of the nucleotide substitution. The minus primer was the pCAT-Basic (−) oligonucleotide 5′CAACGGTGGTATATCCAG3′. The symbol "+" indicates the sense primer, and the symbol "−" indicates the antisense primer. The template for PCR was the *Sma*I-PstI fragment from the 5′ flanking region of the gene (Kuhen and Samuel, 1997). PCR (Saiki *et al.,* 1985) was performed using native Taq DNA polymerase and conditions specified by the manufacturer (Perkin–Elmer). Subclones were sequenced to verify base substitutions.

Transfection and reporter assays

For the transient expression assay of *Pkr* promoter function, U cells (60-mm dishes) at a density of $\sim 5 \times 10^5$ cells per plate were transfected by the DEAE-dextran-chloroquine phosphate transfection method (Luthman and Magnusson, 1983) using 10 μg of the *PKR* promoter:CAT reporter gene plasmid and 5 μg of the internal reference plasmid pRSV2-βgal (generously provided by J. Nevins, Durham, NC). For comparative purposes, the pCAT-Control (Promega) plasmid containing the simian virus 40 promoter and enhancer and the pCAT-Basic promoter-less plasmid were routinely analyzed in all transfection experiments. DNA plasmids used in transfections were purified by cesium chloride equilibrium centrifugation. Purified plasmids were analyzed by agarose gel electrophoresis to verify plasmid integrity. Treatment with IFN was initiated at ~24 h after transfection. For analysis of CAT and β-galactosidase activity, cell cultures were harvested 65 h posttransfection and extracts prepared by repeated freeze-thaw cycles; CAT and β-galactosidase enzyme assays were performed as described (Sambrook *et al.,* 1989). The protein concentration of extracts was determined by the Bradford method (Bio-Rad Laboratories). CAT activity was quantified after thin layer chromatography by direct measurement of the ^{14}C-acetylated chloramphenicol products using a Beckman LS1801 liquid scintillation system to determine the radioactivity associated with the excised product spots localized using an autoradiogram of the TLC plate. Alternatively, the ^{14}C-acetylated chloramphenicol products were quantified directly on the TLC plate using a molecular imager system (Bio-Rad model GS525). CAT activity values, calculated as percentage conversion of [^{14}C]chloramphenicol to the acetylated derivatives, were normalized by β-galactosidase activity to control for variation in transfection efficiency.

Cell maintenance and interferon treatment

Human amnion U cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with fetal bovine serum (Hyclone) at 5% (vol/vol), 100 units of penicillin per milliliter and 100 μg/ml of streptomycin. Where indicated, interferon treatment was with 1000 IU/ml of IFN-/α.

Nuclear extract preparation

Cells were washed two times with ice-cold phosphate-buffered saline (PBS) prior to the addition of ice-cold lysis buffer [STM (20 mM Tris–HC1, pH 7.8; 250 mM sucrose; 1.1 mM $MgCl_2$), 0.2% Triton X-100; protease inhibitor cocktail (as per Sigma's recommendation); 50 mM NaF; 1 mM

sodium orthovanadate (Na_3VO_4); and 1 mM phenylmethylsulfonyl fluoride (PMSF)]. Lysates were transferred to microfuge tubes and incubated on ice for 5 min. Nuclei were separated from the cytoplasm by centrifugation at 14,000 *g* for 2 min at 4°C. Proteins were extracted from the nuclear pellet in 100 μl of high salt buffer (STM, 0.4 M KCl, 5 mM β-mercaptoethanol, 1 mM PMSF, protease inhibitors as a cocktail, and phosphatase inhibitors), and incubated on ice for 10 min during which time the sample was mixed vigorously by vortexing every 2 min for 10 s. Nuclear extracts were obtained by centrifugation at 14,000 *g* for 10 min at 4°C. Protein concentration was determined by the Bradford method (Bio-Rad). Bandshift results were comparable to those obtained using the extraction method of Dignam *et al.* (1983).

Oligonucleotide probes

Single-stranded oligodeoxynucleotides were synthesized in-house using a Millipore Cyclone Plus automated DNA synthesizer or were obtained commercially from Operon. The following were used, double-stranded, as the ^{32}P end-labeled probes or unlabeled competitor oligomers in the gel-shift analyses (mutations are indicated in underlined, boldface type): wild-type KCS, CTGCAGGGAAGGCGGAGTCCAAGG; wild-type ISRE, CCAAGGGGAAAACGAAACTGCAG; wild-type KCS/ISRE, CTGCAGGGAAGGCGGAGTCCAAGGGGAAAACGAAACTGCAG; mutant KCS(mt6A), CTGCAGGGAA**A**GCGGAGTCCAAGG; mutant KCS(mt9T), CTGCAGGGAAGGC**T**GAGTCCAAGG.

Electrophoretic mobility shift assay

For protein-DNA binding reactions, nuclear extracts prepared from untreated or IFN-treated human U cells were incubated (~10 μg of protein) in 25 μl of total reaction mixture containing 1 μg poly dI:dC; 2 mM Tris, pH 7.6; 0.2 mM EDTA; 8 mM NaCl; 0.8% glycerol; 0.3 mM β-mercaptoethanol; and 5 ng of the ^{32}P-labeled oligonucleotide probe. The probe was added last, and the reaction was incubated for 20 min at room temperature. The entire reaction mixture was analyzed by gel electrophoresis, which was carried out using a 5% native polyacrylamide gel with 0.5— TBE that had been prerun at 4°C for 30 min. Electrophoresis was allowed to continue for ~80 min and the gel was then dried and exposed to X-ray film to obtain an autoradiographic image. Quantification of specific gel-shifted complexes was done by using a BioRad GS525 molecular imager system.

Materials

Unless otherwise specified, all materials and reagents were as described previously (Thomis *et al.,* 1992; Tanaka and Samuel, 1994; Kuhen and Samuel, 1997).

ACKNOWLEDGMENT

This work was supported in part by Research Grant AI-20611 from the National Institute of Allergy and Infectious Diseases, National Institutes of Health.

REFERENCES

Barber, G. N., Wambach, M., Wong, M. L., Dever, T. E., Hinnebusch, A. G., and Katze, M. G. (1993) Translational regulation by the interferon-induced double-stranded-RNA-activated 68-kDa protein kinase. *Proc. Natl. Acad. Sci. USA* **90,** 4621–4625.

Benkirane, M., Neurveut, C., Chun, R. F., Smith, S. M., Samuel, C. E., Gatignol, A., and Jeang, K-T. (1997). Oncogenic potential of TAR RNA binding protein TRBP and its regulatory interaction with the RNA-dependent protein kinase PKR. *EMBO J.* **16,** 611–624.

Clemens, M. J., and Elia, A. (1997). The double-stranded RNA-dependent protein kinase PKR: Structure and function. *J. Interferon Cytokine Res.* **17,** 503–524.

Cosentino, G. P., Venkatesan, S., Serluca, F. C., Green, S. R., Mathews, M. B., and Sonenberg, N. (1995). Double-stranded RNA-dependent protein kinase and TAR-RNA binding protein form homo- and heterodimers in vivo. *Proc. Natl. Acad. Sci. USA* **92,** 9445–9449.

Darnell, J. E. (1997). STATs and gene regulation. *Science* **277,** 1630–1635.

Dignam, J. D., Martin, P. L., Shastry, B. S., and Roeder, R. G. (1983). Eukaryotic gene transcription with purified components. *Methods Enzymol.* **101,** 582–598.

Hershey, J. W. B. (1989). Protein phosphorylation controls translation rates. *J. Biol. Chem.* **264,** 20824–20826.

Kadonaga, J. T., Jones, K. A., and Tjian, R. (1986). Promoter-specific activation of RNA polymerase II transcription by Sp1. *Trends Biochem. Sci.* **11,** 20–23.

Kuhen, K. L., and Samuel, C. E. (1997). Isolation of the interferon-inducible RNA-dependent protein kinase Pkr promoter and identification of a novel DNA element within the 5′-flanking region of the human and mouse Pkr genes. *Virology* **227,** 119–130.

Kuhen, K. L., Shen, X., Carlisle, E. R., Richardson, A. L., Weier, H.-U. G., Tanaka, H., and Samuel, C. E. (1996). Structural organization of the human gene (PKR) encoding an interferon-inducible RNA-dependent protein kinase (PKR) and differences from its mouse homolog. *Genomics* **36,** 197–201.

Kuhen, K. L., Vessey, J. W., and Samuel, C. E. (1998). Mechanism of interferon action. Identification of essential positions within the novel 15-base pair KCS element required for transcriptional activation of the RNA-dependent protein kinase *Pkr* gene. *J. Virol.* **72,** 9934–9939.

Kumar, A., Yang, Y.-L., Flati, V., Der, S., Kadereit, S., Deb, A., Haque, J., Reis, L., Weissman, C., and Williams, B. R. G. (1997). Deficient cytokine signaling in mouse embryo fibroblasts with a targeted deletion in the PKR gene: Role of IRF-1 and NF-kB. *EMBO J.* **16,** 406–416.

Kutoh, E., Margot, J. B., and Schwander, J. (1993). Genomic structure and regulation of the promoter of the rat insulin-like growth factor binding protein-2 gene. *Mol. Endocinol.* **7,** 1205–1216.

Lee, T. G., Tang, N., Thompson, S., Miller, J., and Katze, M. G. (1994). The 58000-dalton cellular inhibitor of the interferon-induced double-stranded RNA-activated protein kinase (PKR) is a member of the tetratricopeptide repeat family of proteins. *Mol. Cell. Biol.* **14,** 2431–2442.

Lengyel, P. (1993). Tumor-suppressor genes: News about the interferon connection. *Proc. Natl. Acad. Sci. USA* **90,** 5893–5895.

Look, D. C., Pelletier, M. R., Tidwell, R. M., Roswit, W. T., and Hotzman, M. J (1995). Stat1 depends on transcriptional synergy with Sp1. *J. Biol. Chem.* **270,** 30264–30267.

Luthman, H., and Magnusson, G. (1983). High efficiency polyoma DNA transfection of chloroquine treated cells. *Nucleic Acids Res.* **11,** 1295–1308.

Meurs, E., Chong, K., Galabru, J., Thomas, N. S., Kerr, I. M., Williams,

B. R. G., and Hovanessian, A. G. (1990). Molecular cloning and characterization of the human double-stranded RNA-activated protein kinase induced by interferon. *Cell* **62,** 379–390.

Nguyen, H., Hiscott, J., and Pitha, P. M. (1997). The growing family of interferon regulatory factors. *Cytokine Growth Factor Rev.* **8,** 293–312.

Ortega, L. G., McCotter, M. D., Henry, G. L., McCormack, S. J., Thomis, D. C., and Samuel, C. E. (1996). Mechanism of interferon action. Biochemical and genetic evidence for the intermolecular association of the RNA-dependent protein kinase PKR from human cells. *Virology* **215,** 31–39.

Patel, R. C., Stanton, P. K., and Sen, G. C. (1996). Specific mutations near the amino terminus of double-stranded RNA-dependent protein kinase (PKR) differentially affect its double-stranded RNA binding and dimerization properties. *J. Biol. Chem.* **271,** 25657–25663.

Porter, A. C. G, Chernajovsky, Y., Dale, T. C., Gilbert, C. S., Stark, G. R., and Kerr, I. M. (1988). Interferon response element of the human gene 6–16. *EMBO J.* **7,** 85–92.

Romano, P. R., Garcia-Barrio, M. T., Zhang, X., Wang, Q., Taylor, D. R., Zhang, F., Herring, C., Mathews, M. B., Qin, J., and Hinnenbusch, A. G. (1998). Autophosphorylation in the activation loop is required for full kinase activity in vivo of human and yeast eukaryotic initiation factor 2 alpha kinases PKR and GCN2. *Mol. Cell. Biol.* **18,** 2282–2297.

Saiki, R. K., Scharf, S., Faloona, F., Mullis, K. B., Horn, G. T., Erlich, H. A., and Arnheim, N. (1985). Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* **240,** 1350–1354.

Sambrook, J., Fritsch, E. F., and Maniatis, G. (1989). "Molecular Cloning: A Laboratory Manual" 2nd Ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Samuel, C. E. (1979). Mechanism of interferon action. Phosphorylation of protein synthesis initiation factor eIF-2 in interferon-treated human cells by a ribosome-associated kinase possessing site specificity similar to hemin-regulated rabbit reticulocyte kinase. *Proc. Natl. Acad. Sci. USA* **76,** 600–604.

Samuel, C. E. (1991). Antiviral actions of interferon. Interferon-regulated cellular proteins and their surprisingly selective antiviral activities. *Virology* **183,** 1–11.

Samuel, C. E. (1993). The eIF-2α protein kinases, regulators of translation in eukaryotes from yeast to humans. *J. Biol. Chem.* **268,** 7603–7606.

Sanger, F., Nicklen, S., and Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.

Sen, G. C., and Lengyel, P. (1992). The interferon system. A bird's eye view of its biochemistry. *J. Biol. Chem.* **267,** 5017–5020.

Tanaka, H., and Samuel, C. E. (1994). Mechanism of interferon action. Structure of the mouse PKR gene encoding the interferon-inducible RNA-dependent protein kinase. *Proc. Natl. Acad. Sci. USA* **91,** 7995–7999.

Thomis, D. C., Doohan, J. P., and Samuel, C. E. (1992). Mechanism of interferon action. cDNA structure, expression, and regulation of the interferon-induced, RNA-dependent P1/eIF-2α protein kinase from human cells. *Virology* **188,** 33–46.

Thomis, D. C., and Samuel, C. E. (1992). Mechanism of interferon action. Autoregulation of RNA-dependent P1/eIF-2α protein kinase (PKR) expression in transfected mammalian cells. *Proc. Natl. Acad. Sci. USA* **89,** 10837–10841.

Thomis, D. C., and Samuel, C. E. (1993). Mechanism of interferon action. Evidence for intermolecular autophosphorylation and autoactivation of the interferon-induced, RNA-dependent protein kinase PKR. *J. Virol.* **67,** 7695–7700.

Vilcek, J., and Sen, G. C. (1996). *In* "Fields Virology," 3rd Ed., Vol. 1, pp. 375–399. Lipppincott-Raven, New York.

Werner, M. H., Gronenborn, A. M., and Clore, G. M. (1996). Intercalation, DNA kinking, and the control of transcription. *Science* **271,** 778–784.

Wong, A. H.-T., Tam, N. W. N., Yang, Y.-L., Cuddihy, A. R., Li, S., Kirchoff, S., Hauser, H., Decker, T., and Koromilas, A. E. (1997). Physical association between STAT1 and the interferon-inducible protein kinase PKR and implications for interferon and double-stranded RNA signaling pathways. *EMBO J.* **16,** 1291–1304.